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Plasma Membrane Lipid Alterations Associated with Cold Acclimation of Winter Rye Seedlings (*Secale cereale* L. cv Puma)[1]

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ABSTRACT

Highly enriched plasma membrane fractions were isolated from leaves of nonacclimated (NA) and acclimated (ACC) rye (*Secale cereale* L. cv Puma) seedlings. Collectively, free sterols, steryl glucosides, and acylated steryl glucosides constituted >50 mole % of the total lipid in both NA and ACC plasma membrane fractions. Glucocerebrosides containing hydroxy fatty acids constituted the major glycolipid class of the plasma membrane, accounting for 16 mole % of the total lipid. Phospholipids, primarily phosphatidylcholine and phosphatidylethanolamine with lesser amounts of phosphatidylglycerol, phosphatidic acid, phosphatidylserine, and phosphatidylinositol, comprised only 32 mole % of the total lipid in NA samples. Following cold acclimation, free sterols increased from 33 to 44 mole %, while steryl glucosides and acylated steryl glucosides decreased from 15 to 6 mole % and 4 to 1 mole %, respectively. Sterol analyses of these lipid classes demonstrated that free β-sitosterol increased from 21 to 32 mole % (accounting for the increase in free sterols as a class) at the expense of sterol derivatives containing β-sitosterol. Glucocerebrosides decreased from 16 to 7 mole % of the total lipid following cold acclimation. In addition, the relative proportions of associated hydroxy fatty acids, including 22:0 (h), 24:0 (h), 22:1 (h), and 24:1 (h), were altered. The phospholipid content of the plasma membrane fraction increased to 42 mole % of the total lipid following cold acclimation. Although the relative proportions of the individual phospholipids did not change appreciably after cold acclimation, there were substantial differences in the molecular species. Di-unsaturated molecular species (18:2/18:2, 18:2/18:3, 18:3/18:3) of phosphatidylcholine and phosphatidylethanolamine increased following acclimation. These results demonstrate that cold acclimation results in substantial changes in the lipid composition of the plasma membrane.

The plasma membrane plays a central role in cellular behavior during a freeze/thaw cycle, and lysis or alterations in its semipermeable characteristics is a primary cause of freezing injury (43). In protoplasts isolated from NA[2] rye leaves, injury over the range of 0 to −5°C is a consequence of freeze-induced osmotic contraction, resulting in irreversible endocytotic vesiculation of the plasma membrane which subsequently results in lysis of the protoplasts during osmotic expansion following thawing of the suspending medium (10, 12). Freezing protoplast suspensions to temperatures below −5°C results in dehydration-induced destabilization of the plasma membrane so that protoplasts are osmotically unresponsive during thawing of the suspending medium (10, 13). Loss of osmotic responsiveness is associated with several changes in the ultrastructure of the plasma membrane, including the formation of lateral phase separations, aparticulate lamellae, and hexagonal$_{II}$ configurations (13).

Cold acclimation dramatically alters the behavior of the plasma membrane during freeze-induced osmotic contraction and dehydration. Osmotic contraction of protoplasts from acclimated rye leaves results in the formation of exocytotic extrusions of the plasma membrane (14). At severe levels of dehydration which accompany freezing of the suspending medium below −5°C, lateral phase separations, aparticulate lamellae, and hexagonal$_{II}$ configurations are not observed in acclimated protoplasts (14).

Preliminary studies contrasting the cryobehavior of liposomes prepared from plasma membrane lipid extracts of NA and ACC rye leaves demonstrate that the differential behavior of the plasma membrane in NA and ACC protoplasts is also apparent in the respective liposomes (18). These studies suggest that the differential cryobehavior of the plasma membrane is, at least in part, a consequence of the lipid composition of the plasma membrane.

The involvement of lipid alterations in cold acclimation has been very controversial and remains to be resolved. In large part, the controversy persists because the majority of reports are based on correlative studies of changes in lipid composition and increases in cold hardiness which have been restricted to analyses of whole tissues or crude membrane preparations, rather than the plasma membrane *per se*. Recent studies of the effect of cold acclimation on the lipid composition of highly enriched plasma membrane fractions suggest that changes in lipid composition and thermotropic properties of the plasma membrane accompanying cold acclimation are rather modest (17, 46, 48, 49). However, a comprehensive lipid analysis (ideally at the molecular species level) is required before any conclusions can be reached. Also, the role of lipid compositional changes in the cold acclimation process should be considered from a perspective of freeze-induced alterations in lipid mesomorphism rather than solely from a perspective of membrane "fluidity." Given that cell dehydration is the principal stress that occurs during freezing of cells (43) and that dehydration-induced lamellar-to-hexagonal$_{II}$ phase transitions are associated with destabilization of the plasma membrane (13), it is more appropriate to consider the causal

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[2] Abbreviations: NA, nonacclimated; ACC, cold acclimated; SG, steryl glucoside; ASG, acylated steryl glucoside; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; PA, phosphatidic acid; PI, phosphatidylinositol; *X:Y*, number preceding colon represents the number of carbon atoms in the fatty acid and the number following the colon indicates the number of double bonds present; *X:Y/X:Y*, fatty acids located at the *sn*-1/*sn*-2 positions of the glycerolipid; *X:Y*(h), hyrdoxy fatty acid: C_x, fatty acid or molecular species derived from phospholipid having *X* acyl chain carbon atoms.

relationship between the lipid composition and the cryobehavior of the plasma membrane from a perspective of lyotropic phase transitions—including both dehydration-induced liquid crystalline to gel and lamellar to hexagonal$_{II}$ phase transitions.

Our current objectives are to characterize, in detail, the lipid composition of the plasma membrane and determine the influence of cold acclimation on lipid composition as a first step in providing a mechanistic explanation for the differential cryostability of the plasma membrane.

MATERIALS AND METHODS

Seeds of rye (*Secale cereale* L. cv Puma) were germinated in moist vermiculite and grown for 7 d in a controlled environment (16 h light at 20°C; 8 h dark at 15°C). NA plants were maintained in this environment for an additional 7 to 10 d prior to isolation of the plasma membrane. Plants were acclimated by transferring to a lower temperature (11.5 h light at 13°C; 12.5 h dark at 7°C) for 1 week, followed by exposure to continuous 2°C temperature (10 h light; 14 h dark) for an additional 4 to 6 weeks.

A highly enriched plasma membrane fraction was obtained from the leaves of rye seedlings using a two-phase partition system of polyethylene glycol/dextran following the procedure described by Uemura and Yoshida (45) with only minor modification. The crude microsomal membranes were suspended in the two-phase medium at a final concentration of 6.2% (w/w) polyethylene glycol/dextran in a solution of 500 mM sorbitol, 30 mM NaCl, and 10 mM K_2HPO_4 (pH 7.6). When purified on a continuous (15–45%) sucrose gradient, the plasma membrane enriched fraction contains a single peak of ATPase activity that co-elutes with a peak of glucan synthetase II activity (7). The ATPase was Mg^{2+}-dependent and stimulated (35–45%) by 50 mM KIDA (potassium iminodiacetic acid) at pH 6.5. ATPase activity was inhibited by vanadate but insensitive to KNO_3 (7). No activity was detected for Cyt *c* oxidase or antimycin-insensitive Cyt *c* reductase. Further, the preparation did not contain any detectable levels of Chl.

Lipids were extracted from plasma membrane preparations using the procedure of Bligh and Dyer (2). Total lipid extracts were separated into neutral lipid, glycolipid and phospholipid fractions on silica Sep Pak cartridges (Millipore) using a procedure based on that of Hamilton and Comai (15). Lipid extracts dissolved in 2 ml chloroform:acetic acid (100:1 v/v) were transferred to the cartridge coupled to a glass syringe barrel. Once the sample had entered the packing, residual sample was washed into the column using 2 ml chloroform:acetic acid (100:1 v/v) followed by an additional 10 to 12 ml of the same solvent mixture to elute neutral lipids. Glycolipids were eluted by the sequential addition of 8 ml acetone and 8 ml acetone:acetic acid (100:1 v/v). Phospholipids were eluted using 5 ml methanol:chloroform:water (100:50:40 v/v/v). Following elution, 1.5 ml chloroform and 2 ml water were added to the phospholipid-containing eluant to effect a phase separation and facilitate recovery of phospholipids. TLC of the polar lipid fractions was performed on precoated plates (Silica Gel 60, 0.25 mm layer thickness, EM Reagents) using a mobile phase consisting of chloroform:methanol:acetic acid:water (85:15:15:3.5 v/v/v/v). Individual lipids were identified by co-chromatography with standards and the use of specific spray reagents (23).

Free sterol content was assayed using cholesterol as a standard, as described by Zlatkis and Zak (51). Lipid sugar content was quantified according to Dubois *et al.* (11). Individual glycolipids were assayed in the presence of silica gel following TLC (41). Based on assays of purified SG, ASG (Supelco), and cerebroside (Sigma) and comparison with sterol assays of isolated SG and ASG, this technique was found to be a suitable, reproducible indicator of the relative content of these glycolipids. Lipid phosphorus was determined using the procedure of Bartlett (1) following $HClO_4$ digestion (33). Individual phospholipids separated by TLC were quantified using the method of Rouser *et al.* (42).

Sterols were analyzed by GC following conversion to tertbutyldimethylsilyl derivatives using MTBSTFA (Pierce Chemical) in dry tetrahydrofuran. The derivatives were separated using a 5 m × 0.53 mm HP-1 capillary column (Hewlett Packard) operated at 250°C. Helium (2 p.s.i. head pressure) was used as the carrier gas. Injector and detector temperatures were maintained at 280 and 300°C, respectively. Fatty acid methyl esters obtained following treatment of phospholipids with boron trifluoride/methanol were separated using a 15 m × 0.25 mm fused silica capillary column wall-coated with SP2330 (Supelco) operated at 200°C, 5 p.s.i. He. Injector and detector temperatures were maintained at 250°C. Split injection was used.

Phospholipid molecular species analysis was carried out as previously described (29, 32). The trimethylsilyl derivatives of diacylglycerols obtained by phospholipase C treatment of the individual phospholipid classes were analyzed by GC using a 15 m × 0.25 mm SP 2330 capillary column temperature programmed from 200°C (1 min hold) to 250°C at 20°C min^{-1} (held until termination of run). Injector and detector temperatures were maintained at 280 and 300°C, respectively. Head pressure was maintained at 12 p.s.i. He. Split injection was used. Identification of molecular species was based on comparison of retention times with standards, mass spectra (GC/MS), and comparison to the relative isothermal retention factors reported by Myher and Kuksis (36). Intramolecular positions (sn-1 *versus* sn-2) of the acyl chains were determined by phospholipase A_2 treatment of the phospholipids (32).

The glycolipids (SG, ASG, and glucocerebroside) were chemically degraded to facilitate analysis. Following separation by TLC and elution from the silica gel (using methanol:chloroform:water (100:50:40 v/v/v) and subsequently effecting a phase separation as described for phospholipids eluted from Sep Pak cartridges) the individual glycolipids were refluxed in 2 N methanolic HCl for 4 h (23). Liberated sterols (and fatty acids, in the case of ASG) were extracted using petroleum ether and analyzed by GC (see above). The methanol phase was taken to dryness under vacuum in the presence of solid KOH and the resulting sugars were converted to trimethylsilyl derivatives using Sylon HTP (Supelco) and analyzed by GC (using similar conditions as described for fatty acid methyl esters except that column temperature was maintained at 160°C) (25). Hydrolysis of the glucocerebrosides required longer (18–20 h) refluxing. Methyl esters were extracted using petroleum ether and reacted with Sylon HTP to form *o*-trimethylsilyl-fatty acid methyl esters prior to analysis by GC. Instrument operating conditions were the same as those for fatty acid methyl esters (above). Identification of hydroxy fatty acids was based on comparison of retention times with standards, and mass spectra obtained by GC/MS. Similarities of fragmentation patterns between plasma membrane hydroxy fatty acids and those from bovine cerebrosides (Sigma) suggest that the rye species are 2-hydroxy fatty acids. Protein was assayed using the method of Peterson (39).

RESULTS

The plasma membrane of rye leaves is characterized by a high lipid to protein ratio (~3.4 μmol mg^{-1}) and a lipid composition that is rather unique relative to other plant membranes (Table I). Free sterols, SG, and ASG comprised >50 mol% of the total lipids. The principal glycolipid of the plasma membrane was determined to be glucocerebroside. Phospholipids constituted 32 mol % of the total lipid in NA samples. Cold acclimation resulted in numerous changes in the lipid composition of the plasma membrane. Free sterols increased from 33 to 44 mol %, and SG and ASG decreased from 15 to 6 mol % and 4 to 1 mol %, respectively. Glucocerebrosides decreased from 16 to 7 mol %,

Table I. *Lipid Composition of Plasma Membrane Isolated from Leaves of NA and ACC Rye Seedlings*

Values expressed as mole percent total lipid ± SE of 4 to 7 determinations.

	NA	ACC
	mol %	
Free sterol	32.7 ± 1.7	44.4 ± 2.6
Steryl glucoside	15.1 ± 1.1	5.7 ± 0.6
Acylated steryl glucoside	4.3 ± 0.3	1.1 ± 0.1
Glucocerebroside	16.2 ± 1.2	6.8 ± 0.8
Phospholipid	31.7 ± 2.1	41.9 ± 1.6
	μmol/mg	
Lipid/Protein	3.38 ± 0.4	3.49 ± 0.5

Table II. *Free Sterol Composition of Plasma Membrane Isolated from Leaves of NA and ACC Rye Seedlings*

Values expressed as mean weight percent of free sterols ± SE of 6 to 7 determinations and as mole percent total lipid.

	NA	ACC	NA	ACC
	wt %		*mol% total lipid*	
Cholesterol	1.5 ± 0.2	0.9 ± 0.2	0.5	0.4
Campesterol	26.7 ± 0.7	22.7 ± 0.7	8.7	10.1
Stigmasterol	7.2 ± 0.9	2.8 ± 0.4	2.3	1.3
β-Sitosterol	63.7 ± 0.7	73.5 ± 1.1	20.8	32.6
Others	<1.0	<1.0	<0.3	<0.4
Total mol%			32.7	44.4

Table III. *Sterol Composition of Steryl Glucoside of Plasma Membrane Isolated from Leaves of NA and ACC Rye Seedlings*

Values expressed as mean weight percent of steryl glucoside ± SE of 3 to 4 determinations and as mole percent total lipid. Glucose was established to be the sole sugar moiety of SG.

Sterol Moiety	NA	ACC	NA	ACC
	wt %		*mol % total lipid*	
Cholesterol	7.9 ± 2.4	6.8 ± 1.3	1.2	0.4
Campesterol	23.0 ± 0.7	22.7 ± 1.7	3.5	1.3
Stigmasterol	3.8 ± 0.5	tr–1.0[a]	0.6	<0.1
β-Sitosterol	59.7 ± 0.8	61.4 ± 0.7	9.1	3.5
Others	<5.0	<9.0	<0.7	<0.5
Total mol %			15.1	5.7

[a] tr = trace.

and phospholipids increased from 32 to 42 mol %.

The predominant free sterols were β-sitosterol and campesterol with lesser amounts of stigmasterol and cholesterol (Table II). Following cold acclimation, the proportion of β-sitosterol increased at the expense of the other sterols. Expressed as mol % of the total plasma membrane lipids, the increase in free sterol content is attributable to a pronounced increase (from 21 to 32 mol % of the total lipid) in β-sitosterol.

β-Sitosterol and campesterol were also the principal steryl moieties in SG and ASG (Tables III and IV). Cholesterol- and stigmasterol-containing species were present in lesser amounts. For both SG and ASG, glucose was the only sugar constituent. The major acyl chains of ASG (esterfied at the 6-0 position of glucose) were 16:0 (44%), 18:1 (8%), 18:2 (20%), 18:3 (16%), and 22:0 (5%); however, the precise pairing of acyl chains and sterol moieties was not determined. Thus, each class of ASG (based on the sterol moiety) actually reflects a family of species differing in the acyl chain constituent. Following cold acclimation, the mol % of SG and ASG species containing β-sitosterol and campesterol decreased (Tables III and IV) in proportions

Table IV. *Sterol Composition of Acylated Steryl Glucoside of Plasma Membrane Isolated from Leaves of NA and ACC Rye Seedlings*

Values expressed as mean weight percent of acylated steryl glucoside ± SE of 3 to 4 determinations and as mole percent total lipid. Sugar moiety was glucose. Major acyl chains at 6-O position of glucose included 16:0, 18:1, 18:2, 18:3 and 22:0. Specific pairing of acyl chains and sterols was not established.

Sterol Moiety	NA	ACC	NA	ACC
	wt %		*mol % total lipid*	
Cholesterol	9.9 ± 1.0	7.6 ± 1.9	0.4	0.1
Campesterol	23.3 ± 2.0	25.4 ± 2.4	1.0	0.3
Stigmasterol	3.4 ± 1.8	tr–2.0[a]	0.1	<0.1
β-Sitosterol	56.3 ± 2.3	61.8 ± 3.2	2.5	0.7
Others	<7.0	<4.0	<0.3	<0.1
Total mol %			4.3	1.1

[a] tr = trace.

Table V. *Phospholipid Composition of Plasma Membrane Isolated from Leaves of NA and ACC Rye Seedlings*

Values expressed as mean mole percent lipid phosphorous ± SE of 3 determinations and as mole percent total lipid.

	NA	ACC	NA	ACC
	wt %		*mol % total lipid*	
PI	2.2 ± 1.7	<1.0	0.7	<0.5
PS	4.5 ± 1.4	2.4 ± 1.9	1.5	1.0
PC	46.7 ± 0.6	46.4 ± 0.2	14.8	19.5
PG	5.8 ± 1.8	5.1 ± 0.8	1.8	2.1
PE	34.5 ± 3.3	37.4 ± 4.2	10.9	15.7
PA	5.4 ± 1.3	5.6 ± 0.1	1.7	2.3
Others	<1.0	<2.0	<0.3	<0.8
Total mol %			31.7	41.9

equivalent to the increase in respective free sterols.

The predominant phospholipids were PC and PE with lesser amounts of PG, PS, PA, and PI (Table V). There were substantial increases in the mol % of PC and PE following acclimation, with correspondingly smaller changes in the other phospholipids, reflecting the increase in phospholipids as a class. However, the relative proportions of the individual phospholipid classes did not change significantly. The major fatty acids of the phospholipid fraction included 16:0 (23%), 18:0 (1%), 18:1 (4%), 18:2 (38%), 18:3 (29%), and 22:0 (4%). Although an increase in 18:3 and decreases in 16:0 and 18:2 were observed following acclimation, these changes were not statistically significant.

Following cold acclimation, there were numerous changes in the molecular species of PC and PE (Table VI). The predominant acyl chain pair of PC and PE (16:0/18:2) decreased relative to other species following acclimation, whereas the relative content of 18:2/18:2 (plus 18:1/18:3) and 18:2/18:3 increased. With the exception of 18:1/18:1, less abundant species having two unsaturated acyl chains also increased. Calculation of the values for mol % total lipid revealed more dramatic differences. For both PC and PE, 16:0/18:2 and 16:0/18:3 increased slightly following acclimation. However, with the exception of 18:1/18:1, species containing two unsaturated chains more than doubled. In contrast to PC and PE, PG contained primarily C_{34} species and only traces of C_{36} species. Disaturated (16:0/16:0) PG was present in small amounts and decreased following acclimation (Table VI).

The hydrolysis products of glucocerebrosides included glucose, long chain bases and hydroxy fatty acids. Degradation during hydrolysis prevented reproducible quantitative analysis of the long chain bases. We therefore focused on characterizing the hydroxy fatty acids. Inasmuch as each glucocerebroside molecule contains one fatty acid and one long chain base, a profile of

Table VI. *Molecular Species Compositions of PC, PE, and PG of Plasma Membrane Isolated from Leaves of NA and ACC Rye Seedlings*

Values expressed as mean weight percent diacylglycerol derivatives of parent phospholipid ± SE of 4 to 5 determinations and as mole percent total lipid.

Molecular Species	PC				PE				PG			
	NA	ACC	NA	ACC	NA	ACC	NA	ACC	NA	ACC	NA	ACC
	wt %		*mol %*		*wt %*		*mol %*		*wt %*		*mol %*	
16:0/16:0, 14:0/18:0[a]	0.6 ± 0.1	0.2 ± 0.1	<0.1	<0.1	—[b]	—	—	—	5.0 ± 0.1	1.5 ± 0.3	0.1	<0.1
16:0/16:1, 14:0/18:1	0.6 ± 0.1	0.2 ± 0.1	<0.1	<0.1	0.2 ± 0.1	tr–0.1[c]	<0.1	<0.1	4.9 ± 0.9	0.5 ± 0.1	0.1	<0.1
16:0/18:0	0.5 ± 0.1	0.3 ± 0.1	<0.1	<0.1	0.8 ± 0.1	0.4 ± 0.1	<0.1	<0.1	0.9 ± 0.2	1.6 ± 0.3	<0.1	<0.1
16:0/18:1	5.4 ± 0.6	4.4 ± 0.5	0.8	0.9	2.0 ± 0.6	1.1 ± 0.2	0.2	0.2	5.1 ± 0.1	2.7 ± 0.3	0.1	<0.1
16:0/18:2	38.1 ± 0.7	29.8 ± 1.3	5.8	5.9	46.5 ± 1.5	38.3 ± 1.8	5.3	6.2	47.6 ± 1.7	50.6 ± 1.6	0.8	1.1
16:0/18:3	25.3 ± 0.5	22.9 ± 0.6	3.8	4.5	20.6 ± 0.5	20.9 ± 0.4	2.3	3.4	28.1 ± 1.1	33.7 ± 2.0	0.5	0.7
18:0/18:1	0.6 ± 0.2	0.3 ± 0.1	<0.1	<0.1	—	—	—	—	—	—	—	—
18:1/18:1	1.6 ± 0.1	0.8 ± 0.2	0.2	0.1	1.1 ± 0.1	0.4 ± 0.1	0.1	<0.1	—	—	—	—
18:1/18:2	2.9 ± 0.3	4.3 ± 0.1	0.4	0.8	1.6 ± 0.3	2.2 ± 0.3	0.2	0.3	—	tr–0.5	—	<0.1
18:0/18:3	0.8 ± 0.1	tr–0.5	0.1	<0.1	tr–0.7	tr	<0.1	<0.1	—	—	—	—
18:2/18:2, 18:1/18:3	9.5 ± 0.5	14.9 ± 1.0	1.4	2.8	10.1 ± 1.2	15.1 ± 1.5	1.2	2.3	tr	tr	<0.1	<0.1
18:2/18:3	6.6 ± 0.7	12.7 ± 1.6	1.0	2.4	5.4 ± 0.8	10.5 ± 1.1	0.6	1.6	tr	tr	<0.1	<0.1
20:0/18:2	tr–0.6	0.4 ± 0.1	<0.1	<0.1	0.6 ± 0.1	0.5 ± 0.1	<0.1	<0.1	—	—	—	—
18:3/18:3	2.5 ± 0.2	4.3 ± 0.7	0.4	0.8	2.2 ± 0.1	3.0 ± 0.5	0.2	0.5	—	—	—	—
22:0/18:2	1.3 ± 0.3	1.6 ± 0.2	0.2	0.3	1.5 ± 0.2	2.5 ± 0.3	0.1	0.3	—	—	—	—
22:0/18:3	1.2 ± 0.4	1.8 ± 0.2	0.2	0.3	2.1 ± 0.6	2.7 ± 0.3	0.2	0.4	—	—	—	—
Others	<3	<2	<0.3	<0.2	<5	<3	<0.5	<0.4	<7	<8	<0.2	<0.2
Total mol %			14.8	19.5			10.9	15.7			1.8	2.1

[a] The acyl chains separated by a slash represent the components in the *sn*-1 and *sn*-2 positions, respectively, of the molecular species. [b] — = not detected. [c] tr = trace.

species based on fatty acid chain composition may be constructed. Each of these, however, may represent a family of species containing different long chain bases. Following acclimation, the glucocerebroside fatty acid composition was altered (Table VII). The relative proportion of 24:1 (h) increased substantially whereas that of the other major hydroxy fatty acids decreased. Nevertheless, when expressed on the basis of mol % total lipid, all of the glucocerebroside species declined following acclimation.

DISCUSSION

The lipid composition of the plasma membrane is distinct from that of other plant membranes in several respects. The most notable distinguishing feature is the relatively high content of glucocerebrosides (16 mol % in NA extracts). Although the presence of glucocerebrosides in plant tissues has been documented (37), glucocerebrosides have not been reported in several previous lipid analyses of highly purified plant plasma membrane fractions (17, 49), including that of Puma rye (46). Other studies have demonstrated the presence of ceramide-containing lipids in the tonoplast (34, 47). Recently, glucocerebroside was reported to be a major constituent of both the plasma membrane and tonoplast of mung beans (50). For the rye plasma membrane, the acyl chain composition of glucocerebrosides is also rather unique in that the major acyl component is 24:1 (h). Although hydroxy fatty acids are common to sphingolipids (37), this unsaturated acid and 22:1 (h), a less abundant constituent, have not been previously identified in extracts of plant tissues. These two monounsaturated α-hydroxy fatty acids are, however, constituents of bovine brain cerebrosides.

Another notable characteristic is the very high proportion of free sterols and sterol derivatives (SG and ASG), which together account for >50 mol % of the plasma membrane lipids. Free β-sitosterol is the most abundant lipid species of the plasma membrane. Sterols and sterol derivatives are much less abundant in endomembranes (35) of plant cells, including the tonoplast (34), and are absent from chloroplast membranes (35). The presence of SG and ASG in the plasma membrane is consistent with the intracellular localization of the enzymes mediating the interconversion of sterol and sterol derivatives: UDP-glucose:sterol glucosyltransferase and steryl β-D-glucoside hydrolase activities have been localized to Golgi dictyosomes and plasma membrane fractions (16, 19). As well, the similarities in acyl chain compositions of PC, PE, and ASG are consistent with the suggestion that the conversion of SG to ASG is mediated by an acyl transferase utilizing acyl chains derived from phospholipids (19, 35).

Another distinctive feature of rye plasma membrane is the relatively low proportion of phospholipids (32 mol %). PC and PE have very similar molecular species profiles, both having

Table VII. *Glucocerebroside Acyl Chain Composition of Plasma Membrane Isolated from Leaves of NA and ACC Rye Seedlings*

Values expressed as mean weight percent fatty acid derivative ± SE of 3 determinations and as mole percent total lipid.

Acyl Chain	NA	ACC	NA	ACC
	wt %		*mol % total lipid*	
16:0[a]	1.9 ± 0.2	1.2 ± 0.1	0.4	<0.1
16:0 (h)[b]	6.1 ± 0.1	5.9 ± 0.2	1.3	0.4
18:0 (h)	tr–0.7	tr[c]	<0.1	<0.1
20:0 (h)	3.8 ± 0.4	2.7 ± 0.7	0.7	0.2
21:0 (h)	1.4 ± 0.3	0.9 ± 0.5	0.2	0.1
22:0 (h)	12.8 ± 0.8	7.9 ± 0.7	2.1	0.5
22:1 (h)	8.1 ± 0.2	7.2 ± 0.4	1.4	0.5
23:0 (h)	1.7 ± 0.6	1.5 ± 0.2	0.3	0.1
24:0 (h)	16.3 ± 0.6	9.5 ± 1.4	2.6	0.6
24:1 (h)	43.2 ± 0.5	60.8 ± 3.6	6.9	4.0
Others	<3.0	<3.0	<0.5	<0.5
Total mol %			16.2	6.8

[a] Number preceding colon represents the number of carbon atoms in the fatty acid and the number following the colon indicates the number of double bonds present. [b] (h) = hydroxy fatty acid. [c] tr = trace.

primarily C_{34} and C_{36} species, whereas PG is composed primarily of C_{34} species. Although C_{34} species predominate in PG of chloroplasts, the position of the respective acyl chains is C_{18} at *sn*-1 and C_{16} at *sn*-2 (30). However, positional analyses of plasma membrane PG demonstrated just the opposite, *i.e.* C_{16} at *sn*-1 and C_{18} at *sn*-2. Differences in the acyl chain positioning in PG species of chloroplast and microsomal membranes were also reported in *Dunaliella* (29, 30) and reflect the different specificities of the PG biosynthetic compartments.

Whether PA is an endogenous constituent of the plasma membrane or the product of phospholipase D activity was not determined in this study. Yoshida and Uemura (50) inferred that PA is a constituent of the plasma membrane of mung bean hypocotyls because significant amounts of PA were present in plasma membrane fractions isolated in the presence of several inhibitors of phospholipase D.

The results presented here are in contrast to previous studies (17, 46, 49) which suggest that cold acclimation results in relatively few changes in the lipid composition of the plasma membrane. When the analysis is carried out to the level of the molecular species and the results expressed as a mol % of total lipid, it is evident that the proportion of virtually every lipid component is altered following acclimation. Most notably, free β-sitosterol increased dramatically, whereas SG and ASG species containing β-sitosterol decreased. These observed compensatory changes in free sterol *versus* SG plus ASG suggest an *in situ* interconversion of the lipids during acclimation. Recent studies using reconstituted enzyme preparations have suggested that SG hydrolase activity is modulated by the surrounding lipid microenvironment (22). It has been proposed that glucosylation/deglucosylation of sterols may influence membrane properties, however, little direct evidence is available concerning the influence of SG and ASG on membrane structure and function. In addition to the pronounced changes in sterols, the levels of molecular species of PC and PE having two unsaturated acyl chains doubled following acclimation. The changes observed following cold acclimation were similar to those reported for PC and PE molecular species of *Dunaliella* during acclimation to low growth temperatures (29, 30). A pronounced decrease in the cerebroside content of the plasma membrane following acclimation was also observed, with 24:1 (h)-containing species decreasing by almost 50% and the other species decreasing by 60 to 80%.

Although the principal objective of this study—to determine whether cold acclimation alters the lipid composition of the plasma membrane—has been accomplished, the affirmative results present a more challenging problem: that of elucidating the roles of these observed changes in lipid composition in the process of cold acclimation. The complexity of the lipid composition of the plasma membrane and the numerous changes following cold acclimation preclude the possibility that any simple correlative analysis of the changes will establish their role in the cold acclimation process. Instead, a mechanistic approach must be taken to establish a causal relationship between the changes in the lipid composition of the plasma membrane and the increased cryostability of the plasma membrane.

Following cold acclimation there are several differences in the cryobehavior of the plasma membrane. Freeze-induced osmotic contraction of NA protoplasts results in endocytotic vesiculation of the plasma membrane whereas osmotic contraction results in the formation of exocytotic extrusions of the plasma membrane of ACC protoplasts (10, 12, 13, 43). Severe freeze-induced dehydration results in lamellar-to-hexagonal$_{II}$ phase transitions in the plasma membrane of NA protoplasts but not ACC protoplasts (13). Also, the temperature at which intracellular ice formation occurs in NA and ACC protoplasts is substantially different (9) and is related to mechanical failure of the plasma membrane. In addition, cold acclimation alters the electrical characteristics of the plasma membrane and increases the critical membrane potential that results in electroporation of the plasma membrane (44). Because many of these differences in the behavior of the plasma membrane are also observed in liposomes formed from lipids of NA and ACC plasma membranes (18, 28) (DV Lynch, PL Steponkus, unpublished data), we propose that changes in lipid composition are, in part, responsible for the differential cryobehavior of the plasma membrane. Given the diversity of membrane characteristics that are changed following cold acclimation, it is unlikely that there is any one particular change in the lipid composition that is universally responsible for all of the observed differences. There is, however, a possible commonality between two aspects of plasma membrane cryobehavior—the propensity for endocytotic vesiculation and hexagonal$_{II}$ formation in NA protoplasts—that is a consequence of lipid composition. For example, endocytotic vesiculation requires bilayer fusion which is dependent upon the close approach and subsequent destabilization of bilayers. The formation of hexagonal$_{II}$ structures has been postulated as a critical event in membrane fusion (4, 5) (however, see [20]). Thus, it is possible that endocytotic vesiculation during osmotic contraction and formation of hexagonal$_{II}$ structures during severe dehydration reflect some common properties related to the lipid components of the plasma membrane of NA plants. Exocytotic extrusion of the plasma membrane during osmotic contraction of ACC protoplasts does not involve membrane fusion—possibly because the membrane lipid properties are not disposed to such a destabilization, as reflected in the absence of lamellar-to-hexagonal$_{II}$ phase transitions following severe dehydration.

Lyotropic phase transitions and nonbilayer lipid structures have been reported in dehydrated cell membranes and liposomes (3, 4, 13). Removal of water and subsequent close approach of lipid bilayers is proposed to induce demixing in the region of close approach which facilitates the formation of hexagonal$_{II}$ structures (40). Such a sequence of events may occur in the plasma membrane of NA protoplasts, which exhibit lateral phase separations, aparticulate lamellae, and hexagonal$_{II}$ structures as a consequence of freeze-induced dehydration (13). These structures are not observed in ACC protoplasts. Increased membrane stability may be achieved by altering the lipid composition such that demixing and/or liquid crystalline to gel phase transitions (induced by dehydration) are precluded, thus interrupting the sequence of events leading to hexagonal$_{II}$ structures in the plasma membrane.

The decrease in glucocerebroside and the increases in free sterol and phospholipid following acclimation would be expected to increase membrane stability by preventing the formation of separate phases or domains within the membrane. Studies of the interactions between bovine brain cerebrosides and 1-palmitoyl-2-oleoylphosphatidylcholine have demonstrated that changes in the proportions of these two components alter the phase behavior of the mixture (6). At cerebroside concentrations <15 to 20 mol %, a single mixed liquid-crystalline phase is observed at temperatures above the transition temperature of PC. At higher cerebroside concentrations (20–70 mol %) a fluid phosphatidylcholine-rich liquid crystalline phase and a cerebroside-rich gel phase coexist (6). The presence of sterols influences the phase behavior of cerebroside/phospholipid mixtures. The addition of >40 mol % cholesterol effectively eliminates the order-disorder transition of bovine cerebrosides (38). Similarly, lipid extracts of brain myelin do not display a detectable endothermic transition whereas cholesterol-depleted lipid extracts exhibit a broad transition (24). The strong intramolecular hydrogen bonding between hydroxy fatty acid-containing cerebroside molecules results in tight packing of these lipid species in the bilayer (26) and is influenced by dissolved ions (21). Thus, alteration of the ionic

conditions of the aqueous phase (*e.g.* freeze-induced increase in solute concentration of the unfrozen fraction) could alter the packing in localized domains.

Based on these observations, it can be proposed that the proportions of sterol and glucocerebroside present in NA plasma membrane, (~33 mol % sterol and ~16 mol % glucocerebroside) may, under the conditions of decreasing temperature and increasing dehydration as experienced during freezing, lead to the formation of cerebroside-rich domains and domains enriched in nonbilayer forming phospholipids (*e.g.* PE). The stoichiometry of the sterol and cerebroside components in the plasma membrane following acclimation (~44 mol % and ~7 mol %, respectively) would diminish the probability of the formation of cerebroside domains and minimize the possibility of demixing and subsequent hexagonal$_{II}$ formation. Although the transbilayer distribution of glucocerebrosides has not been determined for rye plasma membrane, it has been established that cerebrosides of myelin are located exclusively in the outer monolayer (27). If such an asymmetric distribution also exists for the rye plasma membrane, it would result in the outer monolayer of the NA plasma membrane containing 32 mol % cerebroside and that of ACC plasma membrane containing 14 mol %. Thus, the potential for formation of cerebroside-rich domains in the outer monolayer would be enhanced in the case of the NA plasma membrane, whereas the higher levels of free sterols and phospholipids coupled with the lower glucocerebroside content in the outer monolayer would decrease the probability of demixing in ACC plasma membrane.

The increase in molecular species of PC and PE containing two unsaturated acyl chains may augment acyl chain unsaturation as a means of altering membrane properties (31). However, di-unsaturated species of phospholipids, especially PE, also have a greater propensity to form nonbilayer structures (3–5). This is an apparent paradox, because hexagonal$_{II}$ structures are not observed in protoplasts of ACC rye protoplasts subjected to a freeze/thaw excursion (13). However, it is known that sterol-phospholipid interactions differ for mixed-acyl chain (*i.e.* saturated/unsaturated) species and di-unsaturated acyl chain species (8). Furthermore, demixing and formation of nonbilayer structures may be precluded by changes in the proportions of sterol, glucocerebroside, and phospholipid in spite of the greater abundance of nonbilayer forming phospholipid species.

Testing these various possibilities requires detailed physical studies of specific lipids and lipid mixtures, as well as investigations of cryobehavior of liposomes composed of defined mixtures of the various lipid components. Detailed knowledge of the lipid composition of the plasma membrane, as reported here, will serve as a foundation for these future studies.

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